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# Embryonic Development of the Light Organ of the Sepiolid Squid *Euprymna scolopes* Berry

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**Abstract.** The sepiolid squid *Euprymna scolopes* maintains luminous bacterial symbionts of the species *Vibrio fischeri* in a bilobed light organ partially embedded in the ventral surface of the ink sac. Anatomical and ultrastructural observations of the light organ during embryogenesis indicate that the organ begins development as a paired proliferation of the mesoderm of the hindgut-ink sac complex. Three-dimensional reconstruction of the incipient light organ of a newly hatched juvenile revealed the presence of three pairs of sacculate crypts, each crypt joined to a pore on the surface of the light organ by a ciliated duct. The crypts, which become populated with bacterial symbionts within hours after the juvenile hatches, appear to result from sequential paired invaginations of the surface epithelium of the hindgut-ink sac complex during embryogenesis. A pair of anterior and a pair of posterior ciliated epithelial appendages, which may facilitate infection of the incipient light organ with symbiotic bacteria, develop by extension and growth of the surface epithelium. The ink sac and reflector develop dorsal to the crypts and together function to direct luminescence ventrally. These two accessory tissues are present at the time of hatching, although changes in their overall structure accompany growth and maturation of the light organ. A third accessory tissue, the muscle-derived lens, appears during post-hatch maturation of the light organ.

## Introduction

The presence in cephalopods of light organs that harbor luminous bacterial symbionts has been reported in at least 26 species from 5 genera of sepiolids (*Euprymna, Inioteuthis, Rondeletiola, Semirossia* and *Sepiola*) and in at least 5 species from 2 genera of loliginids (*Loligo* and *Uroteuthis*) (Herring, 1988). Although the light organs may have evolved independently in these two families (Sepiolidae and Loliginidae), they are all associated with the ink sac, which is located in the center of the mantle cavity. These organs are typically large and in certain species may have a length equal to 30% that of mantle length (Herring *et al.*, 1981). The bacterially produced light emitted from the organ is thought to function in counterillumination, a behavior whereby the animal emits light of the same wavelength and intensity as downwelling light to camouflage its silhouette from predators below (Herring *et al.*, 1981). Although these light organs occupy a substantial portion of the mantle cavity and are important in the animal's behavior, little is known about their embryonic development.

*Euprymna scolopes*, a small benthic squid (average mantle length of adults = 22 mm) indigenous to Hawaii (Berry, 1912; Singley, 1983), maintains luminous bacterial symbionts of the species *Vibrio fischeri* (Boettcher and Ruby, 1990) in a bilobed light organ (Wei and Young, 1989; McFall-Ngai and Montgomery, 1990). The mature light organ of *E. scolopes* and of other closely related sepiolids is embedded in the ventral surface of the ink sac and is composed of several host tissues that include a reflector, a muscle-derived lens, and a core of tissue that houses the bacteria (Herring *et al.*, 1981; McFall-Ngai and Montgomery, 1990). This tissue that contains the symbionts is composed of cavities lined with epithelial cells, in which the bacteria are maintained, and supportive connective and vascular tissue.

Several aspects of the reproductive biology of *Euprymna scolopes* make this species particularly amenable to developmental studies. Adult females, which mature in four to six months (Singley, 1983), are present in the field throughout the year. Mature squids readily mate in cap-

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tivity, and females maintained in the laboratory typically lay clutches of 50 to 200 eggs on pieces of dead coral or on the walls of their tanks (Singley, 1983). Females of *E. scolopes* may lay several clutches of eggs at intervals of several days to a few weeks, unlike many cephalopod species in which the female dies soon after a single reproductive effort. Embryonic development of *E. scolopes* is temperature dependent, and juveniles typically hatch 21–22 days after eggs are layed when maintained at 23°C (Wei and Young, 1989; pers. obs.) and within 20 days at 24°C (Arnold *et al.*, 1972).

The light organ of a newly hatched *Euprymna scolopes* does not contain bacterial symbionts but becomes infected within hours post-hatch by symbiotic bacteria present in seawater that is pumped through the mantle cavity in the normal ventilatory process (Wei and Young, 1989; McFall-Ngai and Ruby, 1991). Symbionts enter the incipient light organ through several pores and, within 24 hours post-infection, populate cavities lined by epithelial cells (McFall-Ngai and Ruby, 1991). During initiation and establishment of the symbiotic association, the light organ undergoes a series of morphogenetic events that results in a fully differentiated structure in which the bacterial symbionts are presumably maintained under steady state conditions (McFall-Ngai and Ruby, 1991; Ruby and McFall-Ngai, 1992). Previous work indicates that the light organ of juveniles remains in a state of arrested morphogenesis until it is exposed to, and inoculated with, a competent strain of symbiotic bacteria (McFall-Ngai and Ruby, 1991; Montgomery and McFall-Ngai, 1991).

Although the general embryonic development of *Euprymna scolopes* has been described (Arnold *et al.*, 1972), development of the light organ has remained undescribed. Light organ development in *E. scolopes* appears similar to that described by Pierantoni (1918) for *Sepiola intermedia* Naef, a closely related species found in the Mediterranean. Pierantoni reported that the light organ rudiment developed from the outer epithelium and underlying tissues of the ink sac-hindgut complex. The present paper provides the first ultrastructural study of cephalopod light organ development and offers a basis of comparison between development in the Hawaiian sepiolid squid, *E. scolopes*, and that in the Mediterranean species, *S. intermedia*, described by Pierantoni 75 years ago. Additionally, by providing an understanding of the embryonic development of the tissues that comprise the juvenile light organ, we will be better able to ascertain the effect of the bacterial symbionts on post-hatch morphogenesis.

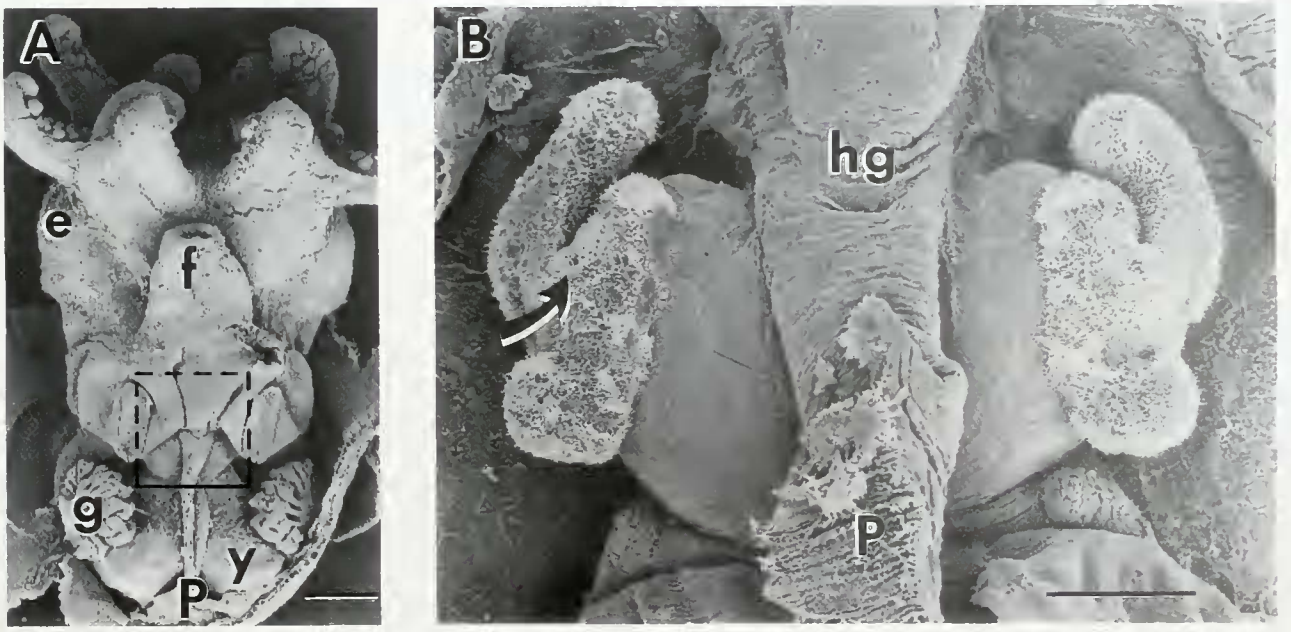


**Figure 1.** Scanning electron micrographs of juvenile *Euprymna scolopes*. A. Ventral view of a newly hatched squid, in which the mantle has been removed to expose the mantle cavity. Most of the light organ (within dashed box) is located inside the funnel so that seawater circulates through the mantle cavity and passes over the light organ before exiting. Scale bar = 250 μm. B. Ventral view of the light organ of a newly hatched squid (both the mantle and funnel have been removed). The lateral face of each side of the light organ is ciliated. Paired anterior and posterior epithelial appendages extend into the mantle cavity. The arrow indicates the direction of flow of seawater over the organ and points to one of the pores, located near the base of the appendages, that open into the interior of the organ. The hindgut lies directly on top of the organ. Bar scale = 100 μm. (e, eye; f, funnel; g, gill; hg, hindgut; y, internal yolk sac; P, posterior).

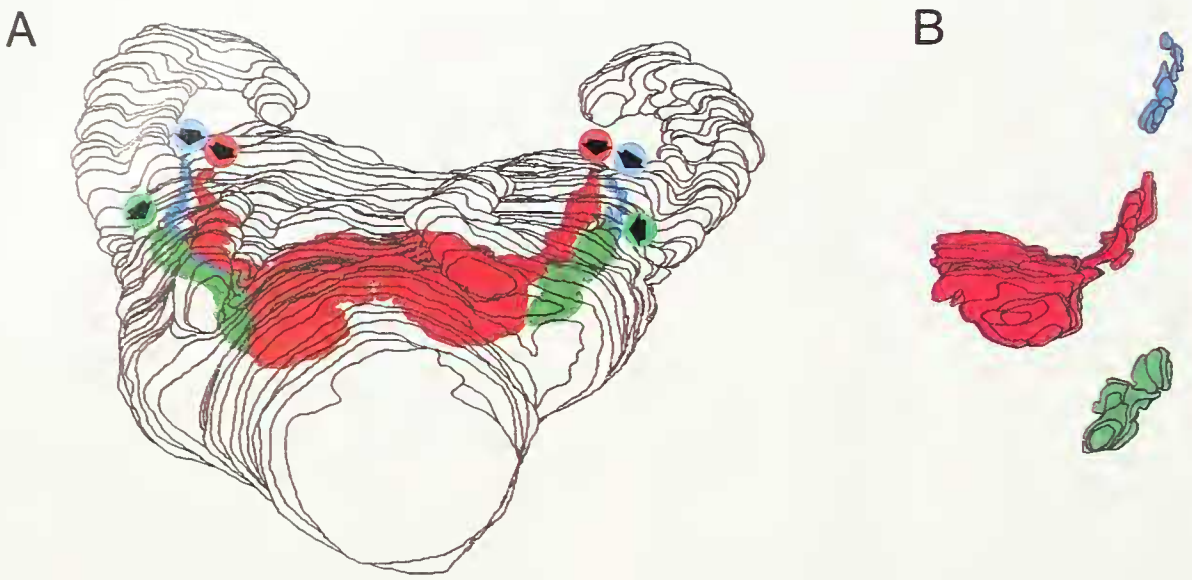


**Figure 2.** Three-dimensional reconstruction of the light organ of a newly hatched juvenile. A. The light organ is shown in ventral view, anterior end at top. The black lines represent the contours of the surface of the organ. Lying within the organ are three pairs of crypts, represented by three separate colors (blue, red, and green). Each crypt communicates with the mantle cavity through a pore (arrows) present on the ventral surface of the organ near the base of the ciliated arms. B. Exploded view of the crypts from one half of the light organ shown at left. The crypts are differently sized with the smallest one, represented in blue, located anterior to the two larger crypts. The medium-sized crypt, represented in green, is located posterior to the other two crypts, whereas the largest crypt, represented in red, is located in the middle.

## Materials and Methods

Adult specimens of *Euprymna scolopes* were collected by dipnet from shallow sandflats in Kaneohe Bay, Oahu in Hawaii throughout 1990 and 1991. The animals were maintained either in running seawater aquaria or in a 250 l recirculating aquarium and on a 12 h light:12 h dark cycle. Mating pairs were placed in individual tanks and females were provided with pieces of dead coral on which to lay eggs. Clutches were removed from the large recirculating aquarium soon after being laid and were placed in smaller aquaria in which the temperature was maintained at 23°C. At various stages of development, embryos were taken from the clutch for analyses.

Embryos and newly hatched juveniles were fixed for light microscopy in 10% formaldehyde in 0.5 *M* sodium phosphate buffer (pH 7.4) at room temperature for 24 h. The embryos were removed from their egg capsule, jelly coats, and chorion with forceps before fixation. Embryos and juvenile squids were fixed for transmission electron microscopy (TEM) in 2.5% glutaraldehyde in 0.1 *M* sodium cacodylate buffer with 0.45 *M* NaCl (pH 7.4) at room temperature for 12 h and post-fixed for 1 h in 1.0% osmium tetroxide in the same buffer as the primary fixative. Following fixation, specimens were rinsed, dehydrated through an ethanol series, cleared with propylene oxide, and embedded in Spurr's resin as previously described (Spurr, 1969; McFall-Ngai and Montgomery, 1990). Ultrathin sections were cut on a Porter Blum MT2-B ultramicrotome with a diamond knife, mounted onto uncoated copper mesh grids, stained with aqueous uranyl acetate and lead citrate, and examined with a JEOL CX-100 transmission electron microscope at 80 kV. Histological sections, 1 to 1.5 $\mu$m in thickness, were cut on the same microtrome and stained with Richardson's stain (Richardson *et al.*, 1960).

Embryos and newly hatched juveniles were fixed for scanning electron microscopy (SEM) in 5% formaldehyde in filtered seawater (FSW) at room temperature for 24 h. Specimens were rinsed in FSW, dehydrated through an ethanol series, and dried by the critical point method. Before sputter coating with gold, the ventral portion of the mantle and any other overlying structures (such as the funnel and gills) were carefully removed to expose the underlying light organ rudiment. Specimens were examined with a Cambridge 360 scanning electron microscope.

Three-dimensional images of light organs from embryos and juveniles fixed for light microscopy were constructed using a computer program (PC3D Three-Dimensional Reconstruction Software, Jandel Scientific, Corte Madera,

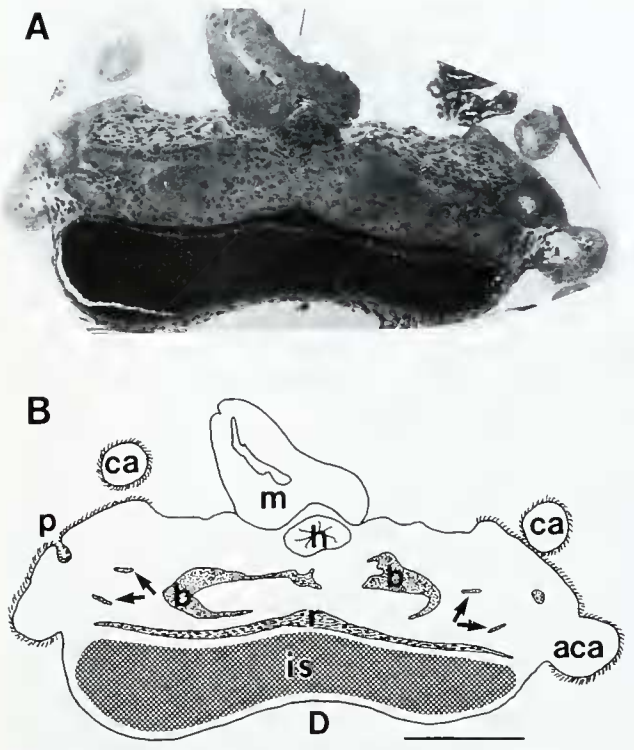


**Figure 3.** Cross section through the light organ of a juvenile 24 h post-hatch. A. Light micrograph of 1.5 μm section. B. Diagram to indicate the relationships among the light organ components. One of the six pores on the ventral surface of the organ is shown; each pore opens into a ciliated duct that leads to an epithelium-lined cavity or crypt. Each crypt (arrows, b) is populated with luminous bacterial symbionts that infected the juvenile squid shortly after it hatched. Scale bar = 100 μm. (aca, anterior ciliated epithelial appendage; b, bacteria; ca, [posterior] ciliated epithelial appendage; D, dorsal; h, hindgut; is, ink sac; m, muscle; p, pore; r, reflector).

California). Serial cross sections 1.0 or 1.5 μm thick of epoxy-embedded material were used for the reconstructions. Either every second or fourth section, depending on the thickness of the sections and the stage of development, was photographed using a Zeiss RA-16 compound microscope with an Olympus photosystem. From the photographs, the outlines of major tissues and structures, such as the surface epithelium, the ink sac, and the lumen of the crypts were digitized with a Numonics 2210 digitizing tablet. The PC3D software stacked the outlines of specific components from each section, producing a three-dimensional (3-D) representation of the light organ rudiment that could be viewed in any orientation. The PC3D software allowed the calculation of the volumes of specific light organ components. The 3-D images shown were plotted on a Hewlett-Packard HP 7475 A plotter.

Light organ length and mantle length (ML) of newly hatched juveniles were measured using an ocular micrometer on a Wild M5 dissecting scope. ML was measured as defined by Young and Harman (1989) for octopodid paralarvae; light organ length as defined here is the distance from the posterior tip of the ink sac to the anterior tip of the ink sac. The developmental stages referred to here (*e.g.*, A25) are those of Arnold *et al.* (1972).

## Results

### *Reproductive biology*

Similar to a previous report (Singley, 1982), individuals of *Euprymna scolopes* under laboratory conditions were nocturnally active and usually remained buried in sand during daylight hours. Adult males were observed mating with females on several nights. Each female laid one to several clutches of eggs, all at night with the exception of one case. In this case, a female layed eggs during daylight hours, which allowed observations of the egg laying process. The female layed the clutch on the side of a piece of dead coral. Each egg was laid down one at a time at approximately one-minute intervals. Between the laying of each egg, the female used her fins to blow sand grains into the space between her arms. Each egg was expelled out the mantle and into the space between the female's arms, where the egg was coated with the sand grains. The egg was slowly moved between the female's extended arms to near the tips; it was then positioned on the clutch. The female appeared to place the eggs fairly randomly on the clutch. If this process is typical of egg laying in this species, then it takes over three hours for a female *E. scolopes* to lay a clutch of 200 eggs.

Following Arnold *et al.* (1972), fertilization was assumed to take place at the time of egg laying, which was designated Day 1. *E. scolopes* individuals reared at 23°C and in a 12 h light:12 h dark regime typically hatched on Day 21. Newly hatched juveniles had an average ML of 1.6 ± 0.1 mm (±S.D., n = 12).

### *General description of the light organ of newly hatched juveniles*

To facilitate an understanding of the embryonic development of the various components of the light organ, the structure of these components as they appear in the light organ of a newly hatched juvenile is described first. The light organ of newly hatched juveniles is located in the center of the mantle cavity, largely within the funnel (Fig. 1A). Based on measurements of live specimens (n = 12), the light organ of newly hatched juveniles averaged 500 ± 45 μm (±S.D.) in light organ width and 390 ± 48 μm (±S.D.) in anterior-posterior length, which was equal to 24 ± 2.5% ML (±S.D.). Scanning electron microscopy revealed that the light organ of the newly hatched juvenile includes a pair of posterior and a pair of anterior ciliated epithelial appendages (Fig. 1B). The anterior appendages are approximately 500 μm in length and 50 μm in width

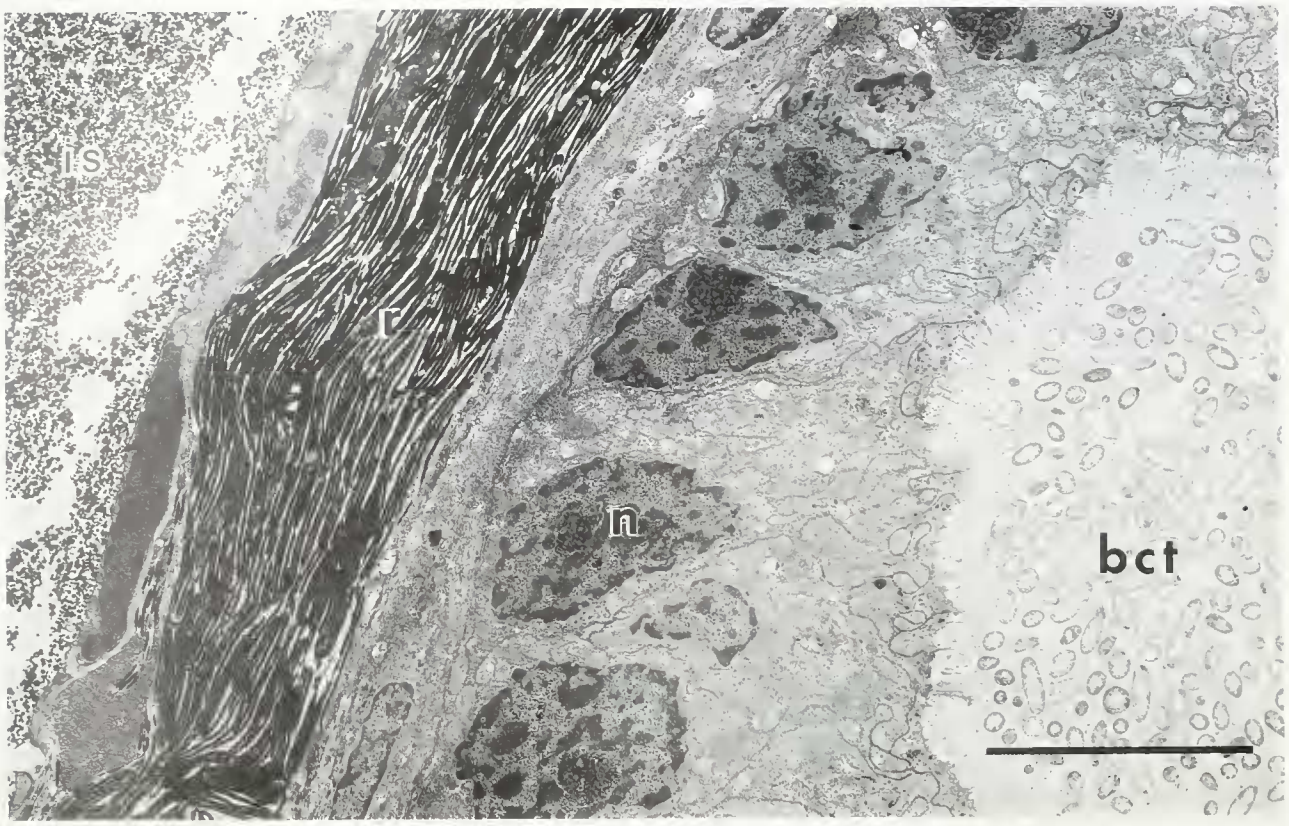


**Figure 4.** Transmission electron micrograph of the light organ of a juvenile 24 h post-hatch. The crypts, populated with bacterial symbionts, are lined with a simple cuboidal epithelium whose apical surface bears an extensive brush border of microvilli. This epithelial cell layer is surrounded by a layer of blood vessels and connective tissue. The reflector is composed of several layers of electron-dense platelets, and is immediately adjacent to the ink sac whose inner surface is composed of a simple squamous epithelium. Scale bar = 10 $\mu$m. (bct, bacteria; is, ink sac; n, nucleus of epithelial cell; r, reflector).

based on measurements of live specimens with a compound microscope. (Tissue shrinkage with SEM is 25–30% in the linear dimension.) TEM revealed that the appendages and surrounding ciliated surface are formed from cells of the surface epithelium, each cell possessing numerous cilia averaging 20 $\mu$m in length and 300 nm in width. Each cilium is surrounded by several microvilli 1–2 $\mu$m in length and 70–115 nm in width, whereas microvilli on adjacent non-ciliated surface epithelium were considerably shorter, ranging from 0.3 to 0.6 $\mu$m in length. On the surface of the light organ are several pores (3 on each side), ranging from 5 to 15 $\mu$m in diameter, that lead into the interior of the organ (Fig. 1B).

Three-dimensional reconstruction of the light organ of a newly hatched juvenile revealed that each pore leads into a separate epithelium-lined cavity or crypt (Fig. 2A); these pores, present on the ventral surface of the organ near the base of the epithelial appendages, connect the crypts to the mantle cavity. The three pairs of crypts, which are roughly bilaterally symmetrical, are different in size. On each side of the incipient light organ the smallest crypt is located most anterior, the medium-sized crypt is most posterior, and the largest crypt is situated between the two smaller ones (Fig. 2B). This pattern of number, size, and position of the crypts was similar in all juveniles examined (n = 14).

The bacterial symbionts populate the extracellular lumen of the crypts following infection of the light organ (Fig. 3). A ciliated duct connects each crypt with a pore on the surface of the organ (Fig. 3). A simple cuboidal epithelium forms the crypt; these epithelial cells contain numerous mitochondria in the region between the basally located nucleus and the apical surface, which, like the appendages, possesses extensive microvilli 1–2 $\mu$m in length (Fig. 4). A cell layer of endothelium and connective tissue underlies the epithelium.

The entire dorsal surface of the bacteria-containing tissue is covered by a reflector and the ink sac (Fig. 4), which function together to control light emission from the organ and to direct the bacterially produced light ventrally. The reflector is composed of iridosomal platelets, which are produced by cells known as iridophores (Arnold, 1967).

**Table I**

*Developmental events of the light organ of* Euprymna scolopes *during embryogenesis**

| Stage | Embryological day | Developmental event |
|---|---|---|
| A25 | 10 | paired lateral mesoderm of hindgut-ink sac complex begins to proliferate |
| A26 | 13 | anterior epithelial appendage begins to form; first pair of crypts begins formation |
| A27 | 15 | anterior and posterior appendages are evident and partially ciliated; epithelial cells of crypts form extensive brush borders; cells toward site of future ducts form cilia; reflector cells begin to differentiate |
| A28 | 17 | second pair of crypts begins formation; epithelial cells forming crypts appear fully differentiated; many reflector cells differentiated; ink cells are functional |
| A29 | 19 | third pair of crypts begins formation |
| A30 | 21 | three crypts are present on each side; entire lateral surface of each side of light organ is ciliated |

* Embryos incubated at 23°C; stages according to Arnold *et al.* (1972).

The reflector in the light organ of a newly hatched juvenile is typically 20 to 30 platelets in thickness. The inner lining of the ink sac is composed of a simple squamous epithelium; this epithelium, with the exception of the ventral surface where the crypts of the light organ are situated, is surrounded by mesodermally derived connective tissue and muscle cells, and an outer epidermally derived epithelium. The lens is not yet differentiated in the newly hatched juvenile.

*Embryonic development*

The light organ begins to develop by stage A25/Day 10, three days after the onset of organogenesis (Table I). At this stage of development, the embryo is 600 μm in total length and only 200 μm in ML. The light organ rudiment appears as a paired proliferation of the mesoderm of the ink sac-hindgut complex (Fig. 5). The ink sac appears as a relatively small outpocketing of the hindgut, in which one or two folds of the ink sac inner epithelium mark the beginning of ink sac gland formation.

*The epithelial appendages.* The epithelial appendages form by extension and growth of the outer surface epithelium of the light organ rudiment. Scanning electron microscopy revealed that the anterior epithelial appendages begin to form by stage A26/Day 12 (Fig. 6A). In addition, a pore on the surface of the light organ rudiment is apparent and signals the onset of formation of the first pair of crypts (Fig. 6B). By stage A27/Day 15, both appendages are evident and both bear cilia, although much of the rest of the lateral surface of the light organ remains without cilia (Fig. 6C). By stage A29/Day 19, the entire lateral surface of each side of the light organ rudiment is covered with cilia. Small blood sinuses and a few mesodermal cells are evident by Day 17 (stage A28) in cross sections of these appendages (Fig. 6E).

*The crypts.* Three-dimensional reconstructions of the light organ rudiments of embryos (one per stage) indicate

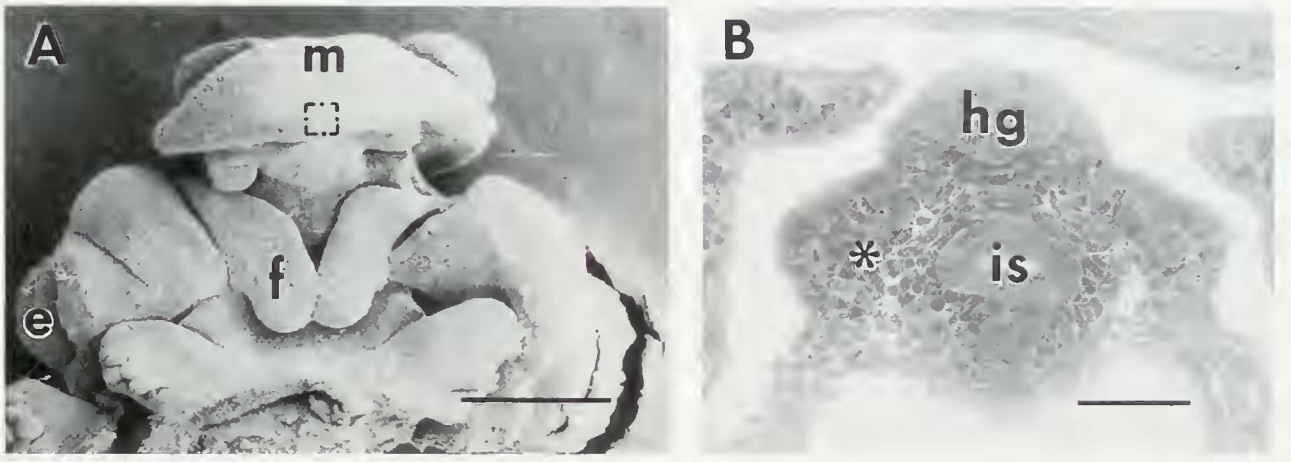


**Figure 5.** *Euprymna scolopes* at embryonic stage A25/Day 10. A. Scanning electron micrograph of the ventral surface of an embryo, animal pole at top. The eyes and funnel folds are prominent. Although the mantle is small relative to the rest of the embryo, the fin primordia and the gill primordia are evident (just visible under the mantle). The dashed box represents the site of the light organ rudiment hidden by the mantle. Scale bar = 100 μm. B. Light micrograph of a histological cross section through the light organ rudiment, which begins development at this stage as a paired proliferation of the mesoderm (*) of the hindgut-ink sac complex. Scale bar = 20 μm. (e, eye; f, funnel fold; hg, hindgut; is, ink sac; m, mantle).

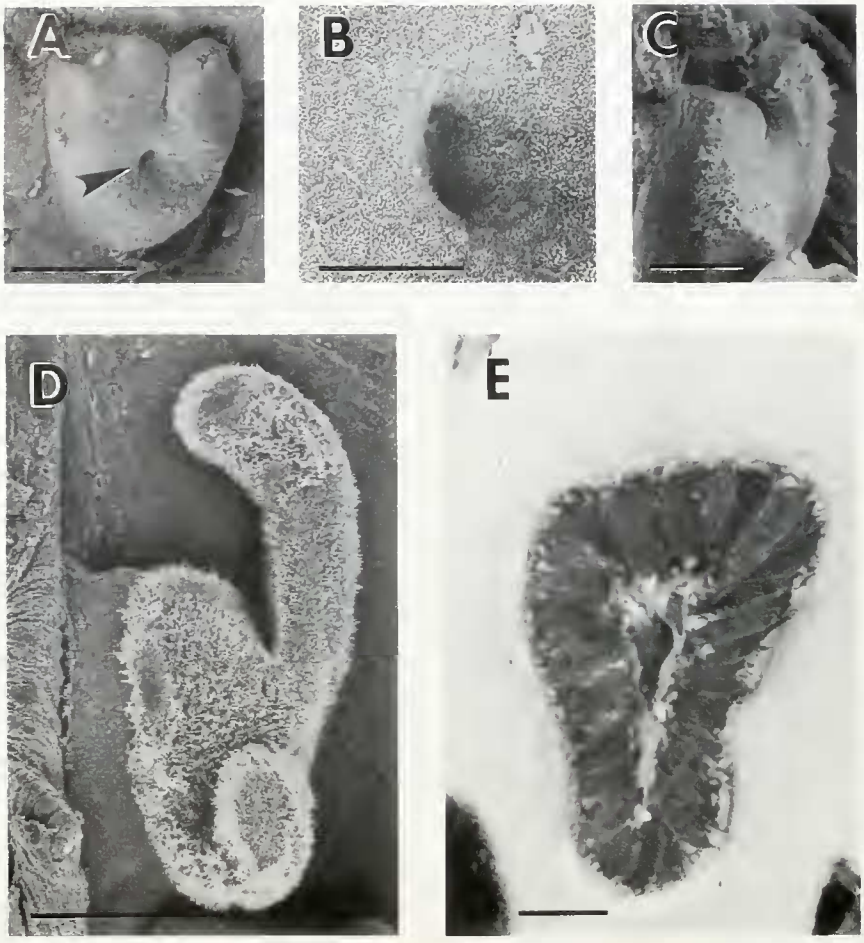


**Figure 6.** Development of the ciliated epithelial appendages on the light organ of *Euprymna scolopes*. A. Scanning electron micrograph (SEM) of one side of the light organ rudiment from a 12-day embryo (stage A26). The anterior appendage is just beginning to form, although the surface is not yet ciliated. The surface epithelium is beginning to invaginate (arrow), leading to the formation of the first crypt. Scale bar = 50 μm. B. High magnification SEM of the pore shown in "A." Scale bar = 10 μm. C. SEM of one side of the light organ rudiment from a 15-day embryo at stage A27. Scale bar = 50 μm. D. SEM of one side of the light organ rudiment from a newly hatched juvenile (stage A30). Scale bar = 100 μm. E. Histological cross section through the epithelial appendage of a 17-day embryo at stage A28. Scale bar = 10 μm.

that the first pair of crypts appears by stage A27/Day 15 (Fig. 7). Two days later (stage A28), the second pair of crypts has begun to form; but, not until stage A29/Day 19 does the third pair appear as two small pits in the surface of the light organ rudiment. The crypts continue to enlarge throughout embryonic development, resulting in a total volume of almost 750 pl in a newly hatched juvenile (Table II); because tissue shrinkage due to fixation has occurred, the actual lengths and volumes of these light organ components may be substantially larger. Measurements of ML of 10 fixed and embedded specimens averaged 1.3 ± 0.1 mm (±S.D.); light organ length of these specimens averaged 310 ± 51 μm (±S.D.), and light organ width averaged 409 ± 48 μm (±S.D.). Thus, compared to measurements of live specimens (see *General description of light organ of newly hatched juvenile*), shrinkage in the linear dimension is approximately 20%. Assuming that the volumes of the crypts and other light organ components shrink accordingly, then the volume calculations may underestimate true volumes by as much as 50%.

The crypts begin formation relatively late in embryonic development and undergo an exponential increase in volume, as compared to light organ length, which increases linearly (Table II; Fig. 8). The apparent growth rate of the crypts is three to four times that of the whole light organ (Fig. 8b). Even when the total volume of the crypts is normalized against ink sac volume to control for variation in size, there is an apparently exponential increase between stage A28/Day 17 and stage A29/Day 19 (Table II).

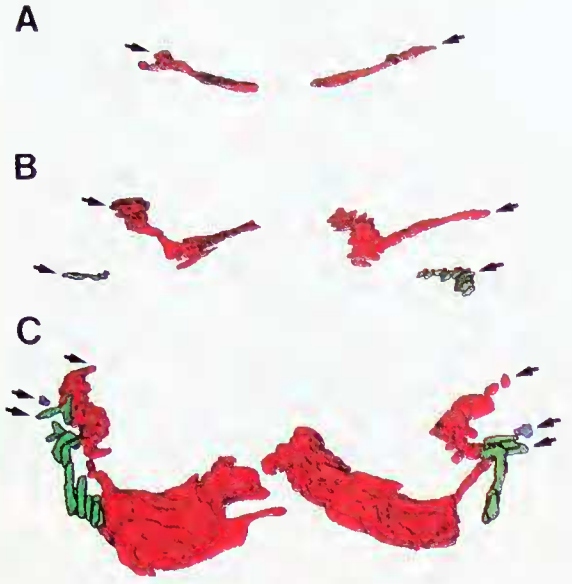


**Figure 7.** Growth in size and number of the crypts of the light organ during embryogenesis as revealed by three-dimensional reconstructions. A. Embryonic stage A27/Day 15. The first pair of crypts (red) is evident as a pair of thin channels. B. Embryonic stage A28/Day 17. The first pair of crypts (red) has enlarged and a second pair (green) posterior to the first is beginning to form. C. Embryonic stage A29/Day 19. The first two pairs of crypts (red, green) continue to enlarge while the third pair of crypts (blue) is just evident as two pits in the surface epithelium. (arrows, pores).

The epithelium lining the crypts is well differentiated by Day 16 (*ca.* stage A28) (Fig. 9). The cells of the epithelium, although columnar in shape, are similar to those from the light organs of newly hatched juveniles. They are polarized with basally located nuclei and an extensive brush border at the apical surface. Numerous mitochondria are located in the apical region of these cells.

*The accessory tissues.* The ink sac is apparent by stage A25/Day 10 as an outpocketing of the hindgut. The iridosomal platelets of the reflector are first evident at stage A26/Day 14 (Fig. 10A, B). The iridophores develop from cells immediately adjacent to the ink sac epithelium (Fig. 10A); the iridosomal platelets appear to form from the condensation of electron-dense material (Fig. 10B). At this stage the epithelial cells lining the inner surface of the ink sac are roughly cuboidal and are not yet producing ink. By stage A27/Day 16 (Fig. 9A), the reflector is several platelets thick, and ink is accumulating. By stage A29/Day 19, two to three days prior to hatching, the inner lining of the ink sac (Fig. 10C) resembles the typical squamous epithelium of the ink sac of a mature animal. The reflector of living specimens, when viewed directly, is visible as a silver lining on the ventral surface of the ink sac, and is 20 to 30 platelets in width, each platelet between 100 and 150 nm in thickness (Fig. 10D). The light organ lens is not yet present when the juvenile hatches.

## Discussion

Embryogenesis in *Euprymna scolopes* results in a light organ that appears primarily structured to facilitate initiation of the symbiosis with *Vibrio fischeri*, whereas the mature light organ primarily functions to maintain the bacterial symbionts and control luminescence. The light organ develops from the recruitment and elaboration of tissues associated with the hindgut-ink sac complex. Tissues that will directly interact with symbiotic bacteria are well developed at the time the juvenile squid hatches, whereas accessory tissues are either not differentiated or not fully elaborated.

### *Early light organ development*

Our observations indicate that light organ development during embryogenesis in *Euprymna scolopes* is similar to light organ development in the closely related sepiolid, *Sepiola intermedia*, as described by Pierantoni (1918). However, our study reveals differences in the order and interpretation of certain developmental events. In both species, the light organ rudiment begins development approximately halfway between the time of fertilization and hatching as a paired proliferation of the mesoderm within the hindgut-ink sac complex. At this stage (A25/Day 10), the ink sac is just beginning to form as a small outpocketing of the hindgut. (For a review of midgut and hindgut development in cephalopods, see Boletzky, 1967, and Raven, 1958.) Pierantoni (1918) reported that the first evidence of light organ development in *S. intermedia* is the presence of two ciliated zones in the epithelium surrounding the visceral sac in the mantle cavity latero-ventral to the anal opening. Although the light organ in *E. scolopes* begins to develop at a similar site, the light organ rudiment has undergone significant development prior to the appearance of cilia. Both the epithelial appendages and the first pair of crypts begin to develop two to three days prior to the appearance of cilia.

### *The epithelial appendages*

The ciliated epithelial appendages comprise a large portion of the incipient light organ of newly hatched *Euprymna scolopes*. Pierantoni (1918) reported the presence of ciliated "zones" on the surface epithelium of the light organ rudiment in *Sepiola intermedia*, although he does not describe their structure or the presence of appendages that extend into the mantle cavity. The epithelial appendages of the light organ of *E. scolopes*, which consist of a longer anterior pair and a shorter posterior pair, may

**Table II**

*Measurements of developing light organs and light organ components based on three-dimensional reconstructions*

| | Embryonic Stage of Development | | | |
|---|---|---|---|---|
| | A27 | A28 | A29 | A30 (newly hatched) |
| Anterior-posterior length of light organ (μm) | 150 | 210 | 275 | 325 |
| Relative light organ length (%) | 46.2 | 64.6 | 84.6 | 100.0 |
| Absolute volume (picoliters) | | | | |
| Total light organ | 1177 | 3920 | 5133 | 13,200 |
| Lumen of crypts, Total | 1.5 | 8.1 | 132.0 | 741 |
| Crypt pair 1 | 1.5 | 7.0 | 123.0 | 622 |
| Crypt pair 2 | 0.0 | 1.1 | 8.5 | 102 |
| Crypt pair 3 | 0.0 | 0.0 | 0.5 | 17 |
| Relative volume (%) | | | | |
| Lumen of crypts, Total (as % of ink sac) | 0.6 | 0.9 | 12.6 | 14.1 |
| (as % of total crypts from newly hatched juvenile) | 0.2 | 1.1 | 17.8 | 100.0 |
| Total light organ | 8.9 | 29.7 | 38.9 | 100.0 |

aid in the infection process (McFall-Ngai and Ruby, 1991). The appendages develop by extension and growth of the outer surface epithelium which originates from the single cell-layered ectoderm.

The cells of tissues that appear to directly interact with the symbiotic bacteria, *i.e.*, the epithelial appendages and the epithelial cells that form the crypts, contain numerous microvilli that are two to three times longer than in epithelial cells immediately adjacent. These microvilli from cells lining the cavities are also two to three times longer than those of secretory epithelial cells of the dorsal epidermis in *Euprymna scolopes* (Singley, 1982), but are similar in size to microvilli in mammalian intestinal epithelium (Fawcett, 1986). Although the cilia on the appendages apparently function to facilitate transport of bacteria toward the pores (see McFall-Ngai and Ruby, 1991), the function of the microvilli is uncertain. It is possible that the microvilli on the cells of the appendages increase cell surface contact with potential symbiotic bacteria and may function in recognition and specificity during the infection process.

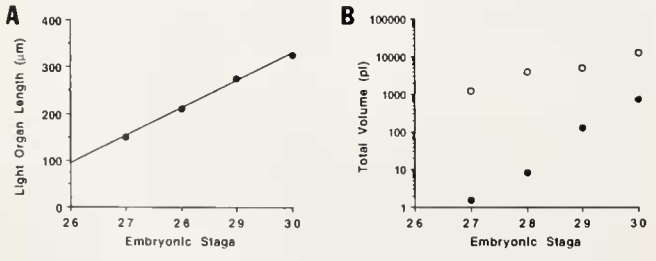


**Figure 8.** A. Changes in light organ length during embryonic development. Measurements based on fixed specimens used for three-dimensional reconstructions. B. Changes in volume of total light organ (open circle) and total crypts within a light organ (closed circles) during embryonic development. Volumes based on calculations from three-dimensional reconstructions (see text).

*The crypts*

Pierantoni (1918) described invaginations of the ciliated surface epithelium of the light organ in *Sepiola intermedia* that give rise to the tubules that eventually house the bacterial symbionts. Our observations of light organ development in *Euprymna scolopes* also indicate that the crypts form from invaginations of the surface epithelium; crypts observed were always joined to a pore on the surface of the organ, even in the earliest stages of development when they were present as simple pits in the outer epithelium (Fig. 7). The pattern (number, size and position) of the crypts in the light organs of newly hatched juveniles is remarkably consistent. The differences in the sizes of the crypts appear to be the result of each pair beginning formation several days apart, so that the largest pair of crypts begins formation approximately eight to ten days (stage A26) before hatching, whereas the smallest pair does not begin formation until just one to two days before hatching (stage A29). Whether the smallest pair, whose volume is little more than 2% that of the largest pair (Table I), serves any function during initiation of the symbiosis is unclear. Nonetheless, if the same growth rate for the crypts is maintained post-hatch, the size of each crypt may double almost daily. Pieratoni (1918) does not report on the numbers of pores or tubules present in the light organs of newly hatched *S. intermedia*. He indicates that the surface epithelium first becomes ciliated before formation of the tubules (*i.e.*, crypts). In contrast, our observations of *E. scolopes* indicate that the first pair of crypts begins to

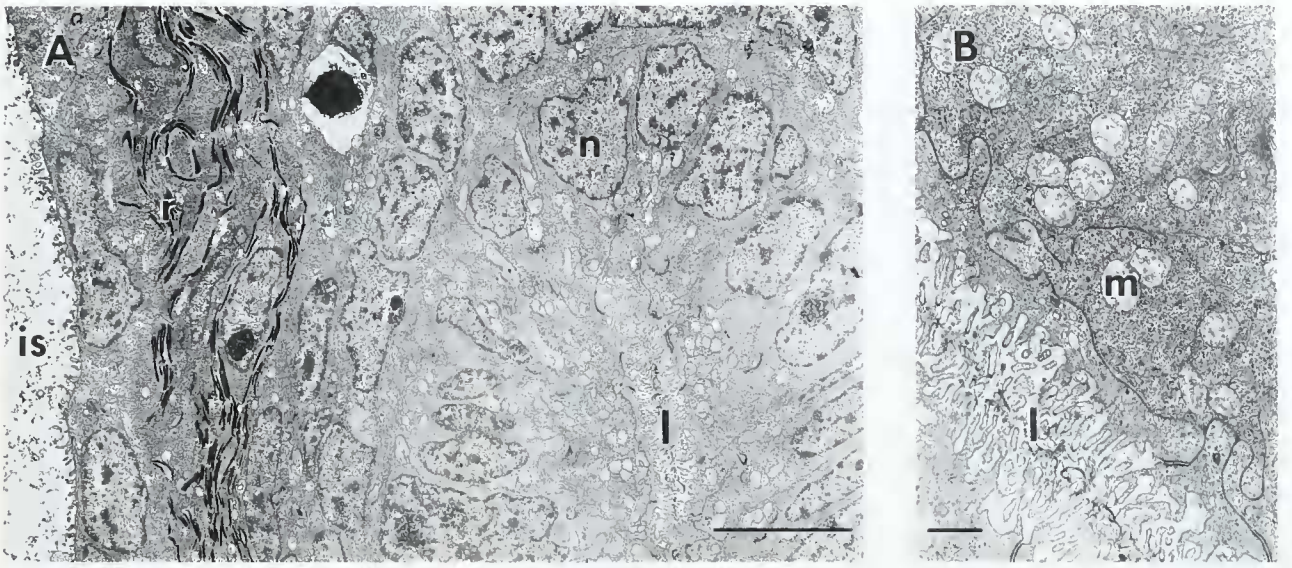


**Figure 9.** Transmission electron micrographs of the light organ rudiment from a 16-day embryo (*ca* stage A28). A. Low magnification view of the epithelium forming the crypts and of surrounding cell layers. The epithelium at this stage is well differentiated, with polarized cells that are similar to those from the light organs of newly hatched juveniles. The nuclei are located toward the basal region of these cells, whereas the apical portion contains numerous mitochondria and bears an extensive brush border. Scale bar = 10 μm. B. High magnification view of the epithelial cells of the same tissue shown in "A" revealing the typical structure of the microvilli and the numerous mitochondria present in the apical portions of these cells. Scale bar = 1 μm. (is, ink sac; l, lumen of crypt; m, mitochondrion; n, nucleus of epithelial cell; r, reflector).

form two to three days before any surface epithelial cells are ciliated.

*The accessory tissues*

Development of the light organ rudiment from the hindgut-ink sac complex results in its central position in the mantle cavity, largely within the funnel. This location may facilitate a rapid infection by maximizing the rudiment's exposure to bacteria in the seawater that circulates through the mantle cavity. The ink sac is also able to function as an accessory structure of the light organ, which uses tissues from the ink bladder and reflective wall to direct and control light emission.

Pierantoni (1918) reported that the reflector in *Sepiola intermedia* develops from muscle tissue. However, our observations of *Euprymna scolopes* indicate that the reflector cells, or iridophores, do not develop from muscle. Pierantoni's observations did not benefit from the use of electron microscopy, and he may have interpreted the laminate structure of the reflector as the striated appearance of muscle cells, which it superficially resembles. The composition of the iridosomal platelets remains unknown, although there is evidence that they are composed largely of protein (Brocco, 1977; Cooper *et al.*, 1990). The wall of the ink sac in many squids and cuttlefishes is highly reflective, so that the ink is camouflaged and not visible through the mantle (Arnold, 1967). Photophores of deep sea squids have also been shown to contain iridiphores that function to reflect bioluminescent light (Arnold *et al.*, 1974). The reflective function is due to the presence of numerous iridophores, similar to those present in the iris and skin (Arnold, 1967). The ink sac wall of *E. scolopes*, a nocturnal animal that remains buried in the sand during the day, is highly reflective only on those portions of the ventral surface that abut the bacteria-containing tissue. However, the ink sac wall does have some reflective material over its entire surface; iridosomal platelets that appear morphologically identical to those in the reflector are present underneath most of the outer epithelium of the ink sac, but are rarely present in numbers greater than two or three. Thus the reflector, which functions to direct the bacterially produced light ventrally, appears to be an augmentation of a primitive feature of the ink sac. The iridosomal platelets, which appear to be membrane-bound, probably form in a manner similar to that described by Arnold (1967) for the iridosomal platelets of the iris and ink sac wall in *Loligo pealei* and *Octopus vulgaris*.

Pierantoni (1918) reported that, in newly hatched *Sepiola intermedia*, the lens has begun to develop from connective tissue underlying the surface epithelium. Previous work on *Euprymna scolopes* (McFall-Ngai and Montgomery, 1990) and other related sepiolids (Herring *et al.*, 1981) has provided evidence that the light organ lens is derived from muscle. Further, although the site of lens

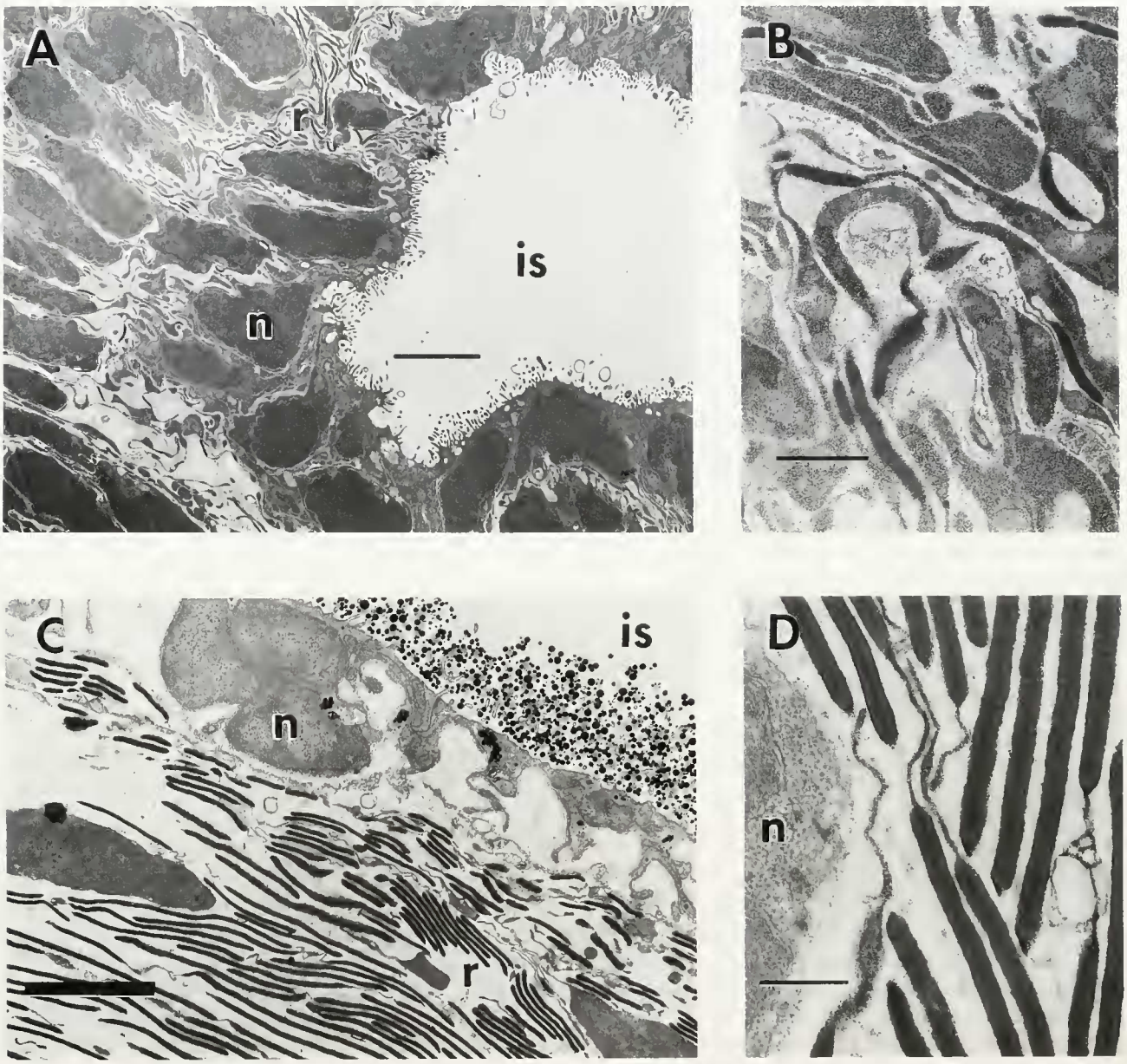


**Figure 10.** Transmission electron micrographs illustrating the development of the accessory tissues of the light organ. A. Low magnification view of the developing reflector and ink sac from the light organ rudiment of a 14-day-old embryo (stage A26). Scale bar = 5 μm. B. High magnification view of the developing reflector platelets from the same specimen shown in "A." Scale bar = 1 μm. C. Low magnification view of the reflector and ink sac from the light organ rudiment of a stage A29/19-day embryo. The reflector is composed of differentiated iridophores containing several stacks of platelets that are roughly aligned parallel with the surface of the ink sac. The typical squamous epithelium of the ink sac is present; the cells are functional, producing and secreting ink into the lumen of the ink gland. Scale bar = 5 μm. D. High magnification view of the reflector platelets shown in "C." Scale bar = 0.5 μm. (is, ink sac; n, nucleus; r, reflector).

development in *S. intermedia* described by Pierantoni is similar in *E. scolopes*, this tissue is mesodermal at the time juvenile *E. scolopes* hatches, and does not begin to differentiate into the lens until seven to ten days post-hatch (Weis *et al.*, 1993).

*Evolutionary relationship with the accessory nidamental gland*

The early developmental stages of the light organ strongly resemble the development of the accessory nidamental gland (ANG), another symbiotic organ in ceph-

alopods (Pierantoni, 1918). Although the development of the bacteria-containing tissue of the light organ appears similar to the early morphogenetic events of the ANG, the light organ does not develop ontogenetically from the ANG as suggested by Herring (1988). Development of these two organs is separated by both time and space. In squid species that have symbiotic light organs, including *Euprymna scolopes* and other closely related sepiolids, mature females have both an ANG and a light organ whereas males have only a light organ (see Buchner, 1965, for review). Further, in *Sepiola intermedia* (Pierantoni, 1918) and in *E. scolopes*, the light organ, which develops from the same set of tissues that gives rise to the hindgut-ink sac complex, begins formation during embryogenesis, whereas the ANG, which is adjacent to but separate from the ink sac, does not begin development until several weeks post-hatch.

However, both the apparent primitive nature of the accessory nidamental gland (ANG) relative to the light organ, and similarities in the development of the two organs suggest that the light organ may have evolved from the ANG. The symbiotic state of the light organ, which is monospecific, differs from that of the ANG, which contains several different strains of pigmented and, typically, non-luminous bacteria (Buchner, 1965). Bloodgood's report (1977) on the ANG of *Loligo pealei* provides evidence that each individual tubule of the ANG is populated with a single strain of bacteria, although the same strain may populate several tubules. Possibly, luminous bacteria established a symbiotic association within specialized tubules of the ANG of one or more ancestral species of squid. Symbiotic luminous organs may have evolved by the separation of luminous bacteria-containing tubules from the ANG, the latter being displaced posteriorly, with a concomitant reduction and specialization of the ink sac as a light organ accessory structure. Such a scenario involving "exaptation" (see Gould and Vrba, 1982) of the ANG and of the ink sac in a common ancestor would explain why all symbiotic luminous organs known in cephalopods are associated with the ink sac. The ANG is closely associated with the ink sac in most species that do not have symbiotic luminous organs. Further, one species of squid, *Rondeletiola minor* (Pierantoni, 1914; Naef, 1923; Herfurth, 1936), possesses a luminous organ and an ANG that are both closely associated with the ink sac, such that the luminous organ and ink sac appear to be embedded within the ANG. Female *Semirossia* (a representative of the sepiolid subfamily Rossiinae) sometimes show a duct uniting the light organs with the ANG instead of a light organ with a duct opening directly into the mantle cavity (Boletzky, 1970). Regardless of whether the light organ evolved directly from the ANG, both organs appear to have developed from similar tissues that are susceptible to interactions with bacteria.

## Conclusions

The light organ of *Euprymna scolopes* begins development from the hindgut-ink sac complex at stage A25/Day 10, halfway between the time of fertilization and hatching. The incipient light organ of newly hatched juveniles appears "poised" for infection by symbiotic bacteria. A large portion of the surface area of the light organ is allocated to structures that most likely aid in the infection process, and in fact these structures regress within a few days following infection when they are presumably no longer needed (McFall-Ngai and Ruby, 1991). Accessory structures, such as the ink sac and reflector, develop during embryogenesis, but change in shape during post-hatch growth and maturation of the light organ. A third accessory structure, the lens, is not yet differentiated in newly hatched juveniles. Thus, the development of the light organ may be divided into three stages that correlate with the state of the symbiotic association: (i) embryonic development, during which the light organ rudiment first forms and the stage is set for initiation of the symbiosis; (ii) early post-hatch development, during which the light organ undergoes morphological and biochemical changes that accompany infection with and subsequent growth of the bacterial symbionts; and (iii) late post-hatch development, which results (within two weeks) in a fully differentiated light organ that primarily functions to maintain the symbiosis and control light emission.

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